METHODS, KITS, AND ANTIBODIES FOR DETECTING PARATHYROID HORMONE

TECHNICAL FIELD

The present invention relates to novel methods and devices for detecting whole or non-fragmented parathyroid hormone (wPTH) in a biological sample. In particular, a novel monoclonal or polyclonal antibody or antibody fragment is used that is specific for a portion of the initial peptide sequence for wPTH which comprises a domain for adenylate cyclase activation, (amino acids 2 to 8), wherein at least four amino acids are part of the reactive portion with the antibody.

BACKGROUND ART

Calcium plays an indispensable role in cell permeability, the formation of bones and teeth, blood coagulation, transmission of nerve impulse, and normal muscle contraction. The concentration of calcium ions in the blood is, along with calcitrol and calcitonin, regulated mainly by parathyroid hormone (PTH). Although calcium intake and excretion may vary, PTH serves through a feedback mechanism to maintain a steady concentration of calcium in cells and surrounding fluids. When serum calcium lowers, the parathyroid glands secrete PTH, affecting the release of stored calcium. When serum calcium increases, stored calcium release is retarded through lowered secretions of PTH.

The complete form of human PTH, (hPTH), is a unique 84 amino acid peptide (SEQ ID NO. 2), as is shown in FIGURE 1. Researchers have found that this peptide has an anabolic effect on bone that involves a domain for protein kinase C activation (amino acid residues 28 to 34) as well as a domain for adenylate cyclase activation (amino acid residues 1 to 7). However, various catabolic forms of clipped or fragmented PTH peptides also are found in circulation, most likely formed by intraglandular or peripheral metabolism. For

example, whole PTH can be cleaved between amino acids 34 and 35 to produce a (1-34) PTH N-terminal fragment and a (35-84) PTH C-terminal fragment. Likewise, clipping can occur between either amino acids 36 and 37 or 37 and 38. Recently, a large PTH fragment referred to as "non-(1-84) PTH" has been disclosed which is clipped closer to the N-terminal end of PTH. (See R. LePage *et alia*, "*A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples* " Clin Chem (1998); 44: 805-810.)

The clinical need for accurate measurement of PTH is well demonstrated. Serum PTH level is one of the most important index for patients with the following diseases: familial hypocalciuric hypercalcemia; multiple endocrine neoplasia types I and II; osteoporosis; Paget's bone disease; primary hyperparathyroidism - caused by primary hyperplasia or adenoma of the parathyroid glands; pseudohypoparathyroidism; and renal failure, which can cause secondary hyperparathyroidism.

Determining circulating biologically active PTH levels in humans has been challenging. One major problem is that PTH is found at low levels, normally 10pg/mL to 40 pg/mL. Coupled with extremely low circulating levels is the problem of the heterogeneity of PTH and its many circulating fragments. In many cases, immunoassays have faced substantial and significant interference from circulating PTH fragments. For example, some commercially available PTH kits have almost 100% cross-reactivity with the non-(1-84) PTH fragment, (see the LePage article).

PTH immunoassays have varied over the years. One early approach is a double antibody precipitation immunoassay found in U. S. 4,369,138 to Arnold W. Lindall *et alia*. A first antibody has a high affinity for a (65-84) PTH fragment. A radioactive labeled (65-84) PTH peptide is added to the sample with the first antibody to compete for the unlabeled peptide. A second antibody is added which binds to any first antibody and radioactive labeled PTH fragment complex, thereby forming a precipitate. Both precipitate and supernatant can be measured for radioactive activity, and PTH levels can be calculated

therefrom.

In an effort to overcome PTH fragment interference, immunoradiometric two-site assays for intact PTH (I-PTH) have been introduced, such as Allegro® Intact PTH assay by the Nichols Institute of San Juan Capistrano, California. In one version, a capture antibody specifically binds to the C-terminal portion of hPTH while a labeled antibody specifically binds to the N-terminal portion of the captured hPTH. In another, two monoclonal antibodies were used, both of which attached to the N-terminal portion of hPTH. (For the purposes of the present invention, the complete form of human PTH is referred to as "whole PTH" or "wPTH" as distinguished from "intact PTH" or "I-PTH" which can include not only wPTH, but also a large PTH fragment cleaved about amino acids 5 to 8.) Unfortunately, these assays have problems in that they measure but do not discriminate between w-PTH and I-PTH. This inability comes to the fore in hyperparathyroid patients and renal failure patients who have significant endogenous concentrations of large, non-whole PTH fragments.

Recently, researchers have made a specific binding assay directed to the large N-terminal PTH fragments. (See. Gao, Ping *et alia* "*Immunochemicalluminometric assay with two monoclonal antibodies against the N-terminal sequence of human parathyroid hormone*", Clinica Chimica Acta 245 (1996) 39-59.) This immunochemiluminometric assay uses two monoclonal antibodies to detect N-terminal (1-34) PTH fragments but not mid-portion PTH fragments or C-terminal PTH fragments. A key factor in the design of these assays is to eliminate any reaction with C-terminal PTH fragments.

DISCLOSURE OF THE INVENTION

The present invention relates to a method for detecting wPTH in a biological sample without detecting the non (1-84) large PTH fragment component of I-PTH, and in particular to a substantially pure monoclonal or polyclonal antibody or antibody fragment

specific for the initial sequence for wPTH which comprises a domain for adenylate cyclase activation, VAL-SER-GLU-ILE-GLN-LEU-MET (SEQ ID No. 1), wherein at least four amino acids in this sequence are part of the antibody reactive portion of the peptide. The method for measuring the amount of wPTH in a sample such as serum, plasma, or blood comprises four general steps which can vary depending upon whether one uses a first antibody or antibody fragment specific for the PTH peptide VAL-SER-GLU-ILE-GLN-LEU-MET (SEQ ID No. 1), wherein at least four amino acids are part of the antibody reactive portion of the peptide either as a signal antibody or a capture antibody in conventional immunoassay formats. Used either as a signal antibody or as a capture antibody, enough antibody is added to bind all w-PTH present. Next, one allows the first antibody to bind to any wPTH present, thereby forming a complex. A specific binding label comprised of a second antibody and a conventional immunoassay label, such as chemiluminescent agents, colorimetric agents, energy transfer agents, enzymes, fluorescent agents, and radioisotopes, is used to label the complex, preferably at the N-terminal end of wPTH, and can be added either substantially simultaneously with the first antibody or subsequent thereto. Finally, one uses conventional techniques to measure the amount of labeled complex, and thereby calculate wPTH levels in the sample. If used as a signal antibody, then the first.

One can also use the present invention to detect the amount of N-terminal PTH fragment present having a complete and functional amino acid sequence from 1 to at least 34. Some researchers are using synthetic N-terminal PTH peptides as a therapeutic treatment to stop bone loss and actually encourage an increase in bone mass. These peptides range from amino acids 1 to at least 34 and on up to 38. The present assay can detect the amount and duration of such synthetic peptides in circulation in a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a diagrammatic view of wPTH.

FIGURE 2 is a diagrammatic view of a wPTH assay using the present antibody as a tracer element.

FIGURE 3 is a diagrammatic view of a wPTH assay using the present antibody as a capture element.

FIGURE 4 is a graph showing a standard curve for a wPTH assay.

FIGURE 6 is a graph comparing a conventional I-PTH assay with the present wPTH assay for healthy normal persons with "normal" PTH values.

FIGURE 6 is a diagrammatic view showing interference from non (1-84) PTH fragments in conventional I-PTH assays.

FIGURE 7 is a graph comparing a conventional I-PTH assay with the present wPTH assay for patients with chronic uremia.

FIGURE 8 is a graph showing the distribution of values for healthy normal persons, patients with primary hyperparathyroidism, and patients with chronic uremia.

BEST MODES FOR CARRYING OUT THE INVENTION

Whole PTH immunoassay

A preferred embodiment of the present invention is an immunoradiometric assay (IRMA), often referred to as a sandwich assay, as shown FIGURES 2 and 3. Elements employed in such an assay (10) include a capture antibody (12) attached to a solid support (14) and a signal antibody (16) having a label (18), attached thereto (20). Typically, one selects a capture antibody that is specific for C-terminal PTH fragments (22), while the label antibody is specific for the initial wPTH peptide sequence which comprises a domain for adenylate cyclase activation (24), as shown in FIGURE 2. However, one could reverse the specificity of these antibodies, as is shown in FIGURE 3.

Alternatively, one could create an immunoassay in which wPTH is either precipitated from solution or otherwise differentiated in a solution, as in a conventional precipitating assays or turbidometric assays. For example, one can use using at least three antibodies to form a precipitating mass. In addition to the initial wPTH sequence antibody and a C-terminal antibody, one can use at least a third antibody which attaches to the mid portion of PTH. The combined mass of wPTH and the at least three antibodies would form a labeled precipitating mass which can be measured by conventional techniques. Another method would be to couple the initial wPTH sequence antibody to colloidal solid supports, such as latex particles.

More specifically, one can create a signal antibody by iodinating 50 micrograms of affinity purified goat anti-(1-8) PTH antibody (Scantibodies Laboratory, Inc., Santee California, U.S.A.) by oxidation with chloramine T, incubation for 25 seconds at room temperature with 1 millicurie of 125-I radioisotope and reduction with sodium metabisulfate. Unincorporated 125-I radioisotope is separated from the 125-1-Goat anti-(1-8) PTH signal antibody by, passing the iodination mixture over a PD-10 desalting column (Pharmacia, Uppsala, Sweden) and following the manufacturers instructions. The

fractions collected from the desalting column are measured in a gamma counter and those fractions representing the 125-1-Goat anti-(1-8) PTH antibody are pooled and diluted to approximately 300,000 DPM (disintegrations per minute) per 100 microliters. This solution is the tracer solution to be used in the whole PTH IRMA.

A capture antibody can be created by attaching affinity purified goat anti PTH 39-84 antibody, (Scantibodies Laboratory, Inc., Santee, California, U.S.A.), to 12 x 75 mm polystyrene tubes (Nunc, Denmark) by means of passive absorption techniques which are known to those of skill in the art. The tubes are emptied and dried, creating solid phase antibody coated tubes.

To conduct an assay of a sample, 200 microliter samples of human serum are added to the solid phase antibody coated tubes. To each tube is added 100 microliters of the tracer solution (labeled goat anti-(1-8) PTH signal antibody). The tubes are incubated at room temperature with shaking for 170 rpm for 20-22 hours. During this time the immunochemical reaction of forming the sandwich of {solid phase goat anti-(39-84) PTH antibody} -- {whole PTH} -- {125-1-goat anti-(1-8) PTH antibody} takes place. Following this incubation, the test tubes are washed with distilled water. Radioactivity on the solid phase, which amount corresponds to the quantity of wPTH present, is measured using a gamma counter. The radioactivity data for the samples is processed by conventional analysis with use of the results from standards and controls and a computer software in order that the concentration of whole PTH in the samples may be ascertained. FIGURE 4 shows a standard curve for such an assay.

Initial whole PTH sequence peptide

In order to make the signal antibody in the above assay, first one makes a synthetic PTH peptide corresponding either to hPTH (Ser - Val - Ser - Glu -Ile - Gln - Leu - Met), rat PTH (Ala -Val - Ser - Glu - Ile - Gln - Leu - Met), or at least four amino acids in the common sequence, absent the first amino acid. The selected peptide can play two roles in making an assay, first as a specific antigenic source for creating a polyclonal antibody or

monoclonal antibody source for signal antibody or capture antibody, and second as part of an affinity purification means for isolating the desired signal antibody or capture antibody.

Briefly, such a peptide can be synthesized on an Applied Biosystems, Inc. (Foster City, California, U.S.A.) Model 431 automated peptide synthesizer employing Fmoc (9-fluoronylmethoxycarbonyl) as the alpha-amino protecting group. All amino acids and solvents are from Applied Biosystems and are of synthesis grade. Following synthesis, the peptide is cleaved from the resin, and side chains are de-blocked, using a cleavage cocktail containing 6.67% phenol, 4.4% (v/v) thioanisole and 8.8% ethanedithiol in trifluoroacetic acid (TFA). The cleaved peptide is precipitated and washed several times in cold diethyl ether. It is then dissolved in water and lyophilized. The crude peptide is subjected to amino acid analysis (Waters PICO-TAG System, Boston, Massachusettts, U.S.A.) and reversed-phase HPLC using a VYDAC (TM) C8 column with 0.1% TFA in water and 99.9% acetonitrile in 0.1% TFA as the mobile buffers. The presence of a single major peak along with the appropriate amino acid composition is taken as evidence that the peptide is suitable for further use.

The resulting peptide is then attached to cross linked agarose beads (Sepharose 4B from Pharmacia, Uppsala, Sweden) according to instructions from the manufacturer. Armed with the initial peptide sequence on a bead, one can affinity purify a polyclonal antibody serum source to isolate the initial sequence antibody for the wPTH immunoassay.

Initial sequence whole PTH antibody

To create an affinity-purified anti-(1-8) PTH antibody, one first uses a selected initial PTH sequence peptide as described above as part of an immunogen for injection into a goat. The peptide can be used either by itself as an injectible immunogen, incorporated into a non PTH peptide having a molecular weight, typically, of between about 5000 and 10,000,000, or as part of the wPTH complete sequence. The immunogen is mixed with an equal volume of Freunds complete adjuvant which is a mixture of light mineral oil and inactivated mycobacterium tuberculosis bacilli. The resulting mixture is homogenized to

produce an aqueous/oil emulsion which is injected into the animal (typically a goat) for the primary immunization. The immunogen dose is approximately 50-400 micrograms. The goats are injected monthly with the same dose of immunogen complex except no mycobacterium tuberculosis bacilli is used in these subsequent injections. The goats are bled monthly, approximately three months after the primary immunization. The serum (or antiserum) is derived from each bleeding by separating the red blood cells from the blood by centrifugation and removing the antiserum which is rich in (1-8) PTH antibodies.

To purify the antiserum for the desired (1-8) PTH antibody, one packs a separation column with the initial PTH sequence peptide bound beads described above, washes the column and equilibrates it with 0.01 M phosphate buffered saline (PBS). The antiserum is loaded onto the column and washed with 0.01 M PBS in order to remove antibodies without the (1-8) PTH specificity. The bound specific goat anti-(1-8) PTH polyclonal antibody is eluted from the solid phase PTH 1-8 in the column by passing an elution solution of 0. 1 M glycine hydrochloride buffer, pH 2.5 through the column. The eluted polyclonal antibody is neutralized after it leaves the column with either the addition of I M phosphate buffer, pH 7.5 or by a buffer exchange with 0.01 M PBS, as is known to those of skill in the art. The polyclonal antibody is stored at 2-8 degrees centigrade.

Comparison between whole PTH and intact PTH assays

The present IRMA assay was compared to a conventional I-PTH immunoassay, the Allegro Nichols Intact PTH assay, (which is commercially available and made by Nichols Institute Diagnostics of San Juan Capistrano, California, U.S.A.), in both PTH normal patients and those suffering from chronic uremia.

FIGURE 5 shows the results for 34 normal human serum samples from healthy subjects which were assayed both by the present wPTH IRMA and the above I-PTH assay. In every case, the level of wPTH detected by the IRMA is lower than that reported by the I-PTH assay, demonstrating the ability of the present IRMA to avoid detecting the interfering

large, non (1-84) PTH fragments detected by the I-PTH assay. FIGURE 6 illustrates how such interference can occur. An N-terminal PTH specific signal antibody which is not specific to the initial PTH peptide sequence, as in the present invention, can detect not only wPTH (as in the upper part of FIGURE 6), but also can detect large, non (1-84) PTH fragments (as in the lower part of FIGURE 6).

A comparison of assay results for 161 chronic uremia patients is shown in FIGURE 7. Serum samples from these patients were measured using the wPTH IRMA and the above I-PTH assay. In every case the wPTH levels are lower than I-PTH values.

Clinical Use

The present wPTH assay has been used in a clinical setting involving 245 persons. The group included 32 persons having normal healthy parathyroid glands, 52 patients with pathologically confirmed primary hyperparathyroidism (1^0HPT), and 161 patients with chronic uremia who are undergoing dialysis on a continuous basis. FIGURE 9 illustrates patient differentating results using the wPTH assay. A person having substantially normal parathyroid hormone function can be differentiated from one having hyperparathyroidism by measuring whole parathyroid hormone levels. Moreover, chronic uremia patients can be differentiated into two groups by measuring whole parathyroid hormone levels in the person and comparing them to normal values, namely, those having substantially normal active parathyroid hormone levels, and those having hyperparathyroidism (or secondary hyperparathyroidism).

The ordinarily skilled artisan can appreciate that the present invention can incorporate any number of the preferred features described above.

All publications or unpublished patent applications mentioned herein are hereby incorporated by reference thereto.

Other embodiments of the present invention are not presented here which are obvious to those of ordinary skill in the art, now or during the term of any patent issuing from this patent specification, and thus, are within the spirit and scope of the present invention.

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